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Distribution of Weedy Red Rice (*Oryza sativa*) Resistant to Imidazolinone Herbicides and its Relationship to Rice Cultivars and Wild *Oryza* Species

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Several weedy red rice populations have evolved resistance to imidazolinone herbicides worldwide. The understanding of the factors related to the herbicide resistance in weedy red rice is important to prevent its occurrence in new areas where imidazolinone-resistant rice cultivars are being used, and to manage the new rice cultivars resistant to herbicides with modes of action other than the acetolactate synthase (ALS)-inhibitors that are being developed. The objectives of this study were to analyze the relationship of weedy red rice populations from southern Brazil with rice cultivars and wild *Oryza* species and to evaluate the occurrence of introgression from rice cultivars and seed migration as the origin of resistance to imidazolinone herbicides in weedy rice. The study was based on 27 weedy red rice populations, seven rice cultivars, and four wild *Oryza* species that were genotyped with 24 simple sequence repeats and three *ALS*-specific single-nucleotide polymorphism markers. A large proportion of the genetic variation of the weedy red rice populations was found within (74%) rather than among populations (26%). The weedy red rice populations were more closely related to the newer rice cultivars that are imidazolinone-resistant than to the older cultivars. The South American native *Oryza glumaepatula* and the other wild *Oryza* species—*Oryza rufipogon, Oryza longistaminata*, and *Oryza glaberrima*—clustered separately from weedy red rice populations, indicating a low likelihood of introgression among weedy red rice and these wild species. Seed migration was an important factor in the genetic structure of the evaluated weedy red rice populations, although gene flow by pollen from resistant cultivars was the principal reason for the spread of herbicide resistance.
Nomenclature: Weedy red rice, *Oryza sativa* L. ORYSA; rice, *Oryza sativa* L. ORYSA; brownbeard rice, *Oryza rufipogon* Griffiths; longstamen rice, *Oryza longistaminata* A. Chev. & Roehr.; and African rice, *Oryza glaberrima* Steud.
Key words: Acetohydroxyacid synthase, Clearfield™, genetic diversity, herbicide resistance.

Weedy red rice is one of the main problems in most of the rice-growing regions of the world because it decreases rice grain yield and milling quality. The main traits related to the invasiveness of weedy red rice are high seed shattering and dormancy, taller plants, red pericarp and high tillering in comparison with cultivated rice (Delouche et al. 2007). However, large variability in these traits occurs, mainly related to plant height resulting in a ''mimicry'' phenotype (Jiang et al. 2012). The effects of weedy red rice on the rice crop are estimated to cost \$274.00 ha^{-1} annually in the state of Arkansas in the United States (Burgos et al. 2008). The conventional chemical control of red rice is limited because both rice and red rice belong to the same species (Chung and Park 2010). The development of imidazolinone herbicide–resistant rice cultivars has allowed selective control of weedy red rice in the rice crop (Shivrain et al. 2010b). This technology has been used across approximately 1.1 million ha in Brazil and the same area in the United States, and is under development in several countries in South and Central America, Central Europe, and Asia. The use of imidazolinone herbicides on imidazolinone-resistant rice cultivars has improved the control of red rice and led to the adoption of better crop management practices. In Brazil, this system started to be used in 2003, and since 2007 these processes resulted in an increase of approximately 40% in the mean rice grain yield in southern Brazil (Roso et al. 2010). Similar benefits have also been observed in other areas where this technology has been used.

The continuous use of imidazolinone herbicides associated with imidazolinone-resistant rice cultivars has resulted in the evolution of weedy red rice that is resistant to imidazolinone herbicides in the United States (Rajguru et al. 2005), Brazil (Menezes et al.

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2009; Roso et al. 2010), and Italy (Busconi et al. 2012; Jiang et al. 2012). The mechanism of resistance has been an altered site of action, and the observed mutations in the *ALS* gene are similar to those present in imidazolinone-resistant rice cultivars (Busconi et al. 2012; Kuk et al. 2008; Roso et al. 2010). Although the outcrossing rate in the genus *Oryza* is low, gene flow has been found to be the predominant source of herbicide resistance in weedy red rice. The assessment of the origin of resistance to imidazolinone in weedy rice in southern Brazil indicated that the spontaneous mutation resulting in the independent evolution of resistance occurred in 1.1% of plants and that gene flow was the major source of resistance (Goulart et al. 2012b). The occurrence of imidazolinone-resistant weedy rice in southern Brazil is a larger problem than in other regions where this technology is being used because of the large cultivated area and the limitations for crop rotation. Previous studies have indicated that the origin of resistance is due to the occurrence of gene flow or independent mutations (Goulart et al. 2012b). However, several factors such as the diversity of climate, crop establishment systems, and use of certified seeds are also related to the dynamic of weedy red rice and require assessment of the population structure to characterize the most suitable measures to prevent the distribution of herbicide resistance to new areas.

The characteristics of gene introgression from cultivated genotypes represent important information that indicates the need for intensification of strategies for the prevention and management of the herbicide resistance in weedy red rice. In situations where crops are grown geographically close to related wild species, gene flow can increase the invasive behavior of wild populations (Ellstrand 2009). Furthermore, the occurrence of herbicide resistance can alter the adaptation of some species (Jiang et al. 2012). For example, a differential germination pattern was observed among different rice cultivars resistant to imidazolinone herbicides with different mutations in the *ALS* gene, indicating a fitness advantage in resistant populations (Goulart et al. 2012a). In addition, weedy red rice individuals resulting from hybridization with wild species were found to have more tillers than the parents (Cao et al. 2009; Song et al. 2004, 2009). The introgression of foreign alleles can also be related to seed migration. For weedy red rice, this is highly important due to its similarity to cultivated rice seeds (Scarabel et al. 2012). These factors can be conjointly evaluated through the analysis of weedy red rice population genetic structure in the area where the infestation occurs in order to establish the management practices required to avoid the distribution of resistance to new areas.

Several genetic and population structure analyses have been carried out with cultivated rice varieties (Borba et al. 2009; Courtois et al. 2012; Zhang et al. 2013). For weedy rice, such studies have also been performed in the United States (Gealy et al. 2012), China (Cao et al. 2006; Zhang et al. 2009), South Korea (Chung and Park 2010), Thailand and Laos (Prathepha 2011), and Italy (Jiang et al. 2012). However, in southern Brazil, where large outbreaks of weedy red rice resistant to imidazolinone occur, these studies are lacking. The analysis of the population structure can also indicate the origin of weedy red rice in a certain region. The ancestry of weedy rice is related to the subspecies *indica* (Londo and Schaal 2007) and *japonica* (Cao et al. 2009), *indica*- and *japonica*-type hybrids (Ishikawa et al. 2005), or hybrids between cultivated rice and *Oryza rufipogon* (Londo and Schaal 2007).

The relationship between weedy red rice from southern Brazil rice fields and other rice cultivars and wild species is limited. In this region, native species of the genus *Oryza* are not present, but the wild species *Oryza glumaepatula* Steud. is native to the Amazon forest and western Brazil (Brondani et al. 2002). This species is diploid and has the same genome type as cultivated rice and is one of the species most prone to hybridize with *Oryza sativa* (Lentini and Espinosa 2005), which indicates the possibility of introgression between this species and weedy red rice. The predominant self-fertilization of the genus *Oryza* is related to the large genetic diversity among geographically distinct populations with some degree of admixtures (Gealy et al. 2012; Reagon et al. 2010). In weedy red rice, this fact could be attributed to the introgression of alleles from wild species of the genus *Oryza* (Londo and Schaal 2007), but more recent and higher-resolution studies in Asia (Jiang et al. 2012) and the United States (Cao et al. 2006, Thurber et al. 2013) have found that weedy rice is originated from dedomestication events from cultivated rice. The possibility of hybridization between *Oryza* species contributes to the maintenance of genetic variation of weedy red rice and favors its persistence in virtually all areas of rice cultivation through rapid adaptation to different climatic conditions and agronomic practices (Reagon et al. 2010; Ziska et al. 2012). Despite the large problem of weedy red

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Table 1. Geographical information for the 27 weedy red rice populations from southern Brazil.

Population	Location	Geographical coordinates	
1	Arroio Grande 1	32.216667 S	53.010000 W
2	Arroio Grande 2	31.999444 S	52.626667 W
3	Arroio Grande 3	32.407222 S	52.901111W
4	Santa Margarida do Sul 1	29.683333 S	53.800000 W
5	Santa Margarida do Sul 2	30.189444 S	54.107778 W
6	São Gabriel 1	30.491944 S	54.494167 W
7	São Gabriel 2	30.500278 S	54.393333 W
8	São Gabriel 3	30.237500 S	54.361111 W
9	São Gabriel 4	30.217778 S	54.565278 W
10	Cacequi 1	29.958889 S	54.905556 W
11	Cacequi 2	29.936667 S	54.909167 W
12	Cacequi 3	29.867500 S	54.772500 W
13	Cacequi 4	29.885278 S	54.877500 W
14	Cacequi 5	29.862222 S	54.133889 W
15	Cacequi 6	29.812222 S	54.022222 W
16	Santo Antônio da Patrulha 1	28.978888 S	50.430555 W
17	Santo Antônio da Patrulha 2	29.919444 S	50.618333 W
18	Campo Bom	29.701666 S	51.048888 W
19	Manoel Viana 1	29.613333 S	55.484444 W
20	Harmonia	29.976666 S	56.162777 W
21	Manoel Viana 2	29.565555 S	55.496388 W
22	Alegrete	29.381388 S	55.610277 W
23	Caverá	30.433611 S	56.190833 W
24	Agudo 1	29.757777 S	52.965555 W
25	Agudo 2	29.521111 S	53.314722 W
26	Agudo 3	29.613611 S	53.320555 W
27	Agudo 4	29.827222 S	53.326111 W

rice resistant to imidazolinone herbicides from southern Brazil, knowledge concerning its genetic diversity and population structure is limited. The objectives of this study were to evaluate the relationship of weedy red rice populations in southern Brazil in relation to rice cultivars and wild *Oryza* species and to assess the occurrence of introgression from rice cultivars and seed migration as the origin of resistance to imidazolinone herbicides.

Materials and Methods

Plant Material and Sampling. The plant material consisted of 27 weedy red rice populations from rice paddy fields in Rio Grande do Sul state, southern Brazil, collected in the 2009 to 2010 season. Each population was composed of 20 panicles of 20 individual plants. Samples were collected in rice paddy fields with suspected presence of weedy red rice resistant to the herbicides imazethapyr and imazapic. The sampling was performed following a "W" shape walking in each field intended to randomly select representative plants. The populations were identified based on region and county location and the geographical coordinates of the sampling sites were obtained using a global positioning system (Table 1). All the sampled populations had red pericarp. Moreover, the imidazolinone-resistant cultivars IRGA 422 CL, PUITA INTA CL, and SATOR CL; imidazolinone-susceptible *japonica*-type cultivar IAS 12-9 FORMOSA; the *indica*-type cultivars EL PASO L144, BR-IRGA 410, and IRGA 417; the wild *Oryza* species *Oryza longistaminata* and *O. rufipogon*; the cultivated species *Oryza. glaberrima*; and the South American native *O. glumaepatula* were included in the analysis. Three plants per rice cultivar, wild species, and O. *glaberrima* were used.

DNA Extraction and Simple Sequence Repeats (SSR) Amplification. Seeds from individual plants were germinated in a germination chamber and subsequently transplanted into pots containing 300 ml of soil from paddy fields. Samples of approximately 150 mg leaf tissue were collected when plants had four leaves. Genomic DNA extraction was performed by a standard cetyltrimethylammonium bromide method (Doyle and Doyle 1987). Polymerase chain reaction (PCR) amplification was performed with 24 SSR markers (Table 2) labeled with fluorescent dyes and organized into four panels as described in Borba et al. (2009). These markers were

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Table 2. Single sequence repeat (SSR) marker information and high allele frequency (HAF), allele per locus (A), alleles per polymorphic locus (P), expected heterozygozity or genetic diversity (He), observed heterozygosity (Ho), polymorphic information content (PIC), and inbreeding coefficient (*f*), based on 27 weedy red rice populations.

SSR	SSR motif	Chromosome	HAF	A	P	He	Ho	PIC	*f*
4653	$(AAG)_{25}$	12	0.47	9	9	0.68	0.15	0.63	0.78
OG106	$(CT)_{27}$	9	0.31	13	13	0.78	0.08	0.75	0.90
RM103	$(GAA)_5$	6	0.54	5	5	0.50	0.07	0.38	0.86
RM204	$(CT)_{44}$	6	0.76	9	9	0.39	0.07	0.35	0.83
RM257	$(CT)_{24}$	9	0.43	16	16	0.72	0.13	0.68	0.82
RM38	$(GA)_{16}$	8	0.46	8	8	0.59	0.08	0.51	0.86
OG44	$(CT)_4$-23bp-$(CT)_{22}$-$(GT)_4$-$(GC)_6$	3	0.91	7	7	0.17	0.13	0.16	0.21
RM171	$(GATG)_5$	10	0.62	7	7	0.52	0.09	0.45	0.82
RM229	$(TC)_{11}$ $(CT)_5C_3$ $(CT)_5$	11	0.51	7	7	0.66	0.17	0.62	0.75
RM231	$(CT)_{16}$	3	0.43	6	6	0.67	0.15	0.60	0.78
RM287	$(GA)_{21}$	11	0.45	9	9	0.73	0.11	0.70	0.84
RM7	$(GA)_{19}$	3	0.49	6	6	0.64	0.24	0.57	0.62
OG10	$(CT)_{29}$	9	0.36	13	13	0.77	0.11	0.74	0.86
RM14	$(GA)_{18}$	1	0.32	19	19	0.81	0.13	0.78	0.84
RM210	$(CT)_{23}$	8	0.44	12	12	0.70	0.08	0.66	0.88
RM222	$(CT)_{18}$	10	0.37	9	9	0.72	0.09	0.67	0.87
RM253	$(GA)_{25}$	6	0.37	11	11	0.73	0.09	0.69	0.88
RM309	$(GT)_{13}$	12	0.45	3	3	0.62	0.08	0.54	0.88
RM11	$(GA)_{17}$	7	0.62	16	16	0.58	0.14	0.55	0.76
RM207	$(CT)_{25}$	2	0.38	16	16	0.72	0.08	0.68	0.89
RM248	$(CT)_{25}$	7	0.34	17	17	0.78	0.25	0.75	0.68
RM252	$(CT)_{19}$	4	0.39	28	28	0.82	0.11	0.81	0.87
RM263	$(CT)_{34}$	2	0.40	11	11	0.70	0.10	0.65	0.86
RM55	$(GA)_{17}$	3	0.40	12	12	0.67	0.10	0.61	0.85
Mean			0.47	11.21	11.21	0.65	0.12	0.61	0.82

selected in a previous study for polymorphism, consistency, and genome distribution based on the analysis of a large number of rice genotypes (Borba et al. 2009). The PCR was performed in a final volume of 5 µl containing 3 ng DNA, the optimum concentrations of each pair of forward and reverse primers (Borba et al. 2009), 2.5 µl Master Mix of Multiplex PCR Kit (Qiagen, Valencia, CA 91355) and 0.5 µl Qsolution Multiplex PCR Kit (Qiagen). The PCR amplifications consisted of 5 min at 94 C; 40 cycles of 60 s at 94 C, 45 s at 56 C, and 1 min at 72 C; followed by 10 min at 72 C. The internal sizing standard GS500 ROX (Applied Biosystems, Foster City, CA 94404) was used for determining the size of the fragments. The fragments were analyzed on a microcapillary sequencer ABI 3100 DNA Analyzer (Applied Biosystems) and alleles were identified by GeneMapper software version 3.5 (Applied Biosystems). The analyses were performed for each of the 20 plants of each population.

Data Analyses. The mean number of alleles; expected heterozygosity, also called gene diversity of Nei (1978); observed heterozygosity and polymorphic information content (PIC) described by Botstein et al. (1980); and the coefficient of inbreeding and genetic distance matrix of Nei (Nei et al. 1983) were estimated for each population and for each SSR marker using the software Power Maker 3.25 (Liu and Muse 2005). The phylogenetic distribution of the weedy red rice populations was evaluated through the genetic distance matrix and a dendrogram was generated based on the unweighted pair group method (UPGMA) using the software MEGA 4 (Tamura et al. 2007). The genetic relationship between individuals of each population was obtained by the genetic distance matrix of Nei (1978) between pairs of individuals, using the software Power Maker 3.25 (Liu and Muse 2005). Based on this matrix, the principal component analysis (PCA) was performed by the software GenAlEx 6 (Peakall and Smouse 2006). In addition, the correlation between genetic and geographic distance among populations was performed using the Mantel test (1967) with 9,999 permutations, by the software GenAlEx 6 (Peakall and Smouse 2006). The analysis of molecular variance (AMOVA) was performed by software GenAlEx 6.

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The population structure analysis and the identification of admixed ancestry individuals were carried out with the software Structure (Pritchard et al. 2000), where each individual was assigned a specific number of clusters (K) based on inferred allele frequencies in each population. The optimal number of genetic clusters was identified by an ad hoc ΔK parameter based on independent runs of K = 2 to 10 as described by Evanno et al. (2005). All runs were iterated for 200,000 Markov chain Monte Carlo sampling steps following 30,000 burn-in steps. The genetic composition assignment of each individual was indicated by colors related to each K group.

Imidazolinone Resistance Analysis. The weedy red rice populations were phenotyped and genotyped for resistance to the herbicide imazethapyr. The same plants used for the molecular analysis were sprayed with the herbicide imazethapyr at 106 g ha^{-1} at the four-leaf-stage. Herbicide injury was evaluated at 25 d after application using a visual scale ranging from 0% for plants without herbicide symptoms to 100% for complete plant death compared with untreated plants and resistant and susceptible standards. Populations with more than 85% chlorotic leaves and a reduction in plant growth compared with the resistant cultivar were graded as a low level of resistance (L). A medium level of resistance (M) was scored when plants had 15 to 84% chlorotic leaves and a partial reduction in growth. A high level of resistance (H) was graded for populations with less than 15% chlorotic leaves.

The *ALS* gene mutations related to imidazolinone resistance were accessed for all 20 plants of each population via the single nucleotide polymorphism (SNP) markers described by Roso et al. (2010). The markers identified the occurrence of the *ALS* gene mutations $Ala_{122}Thr$, $Ser_{653}Asn$, and $Gly_{654}Glu$, which are responsible for imidazolinone resistance in all herbicide-resistant rice cultivars in southern Brazil. The PCR reactions were carried out with 50 ng DNA, 0.166 mM of each nucleotide primer (forward and reverse), 0.166 mM of each deoxynucleotide triphosphate, 0.2 U Taq DNA polymerase, and 1× buffer of 1.5 mM $MgCl_2$, 1.3 µl 100% dimethyl sulfoxide in a total volume of 15 µl. The amplifications underwent 5 min of denaturation at 94 C; 35 cycles of 45 s at 94 C, 45 s at 55, 1 min at 72 C; and 10 min at 72 C. The PCR products were visualized on agarose gel (3%) stained with ethidium bromide at a concentration of 0.02 µl ml^{-1} for 120 min at 110 V in 0.5× Tris/Borate/EDTA buffer (40 mM Tris, 1 mM EDTA, pH = 8.0).

Results and Discussion

Genetic Diversity among Weedy Red Rice Populations. The SSR markers detected 269 alleles and the mean number of alleles per locus was 11.21, ranging from three to 28 for markers RM309 and RM252, respectively (Table 2). This value is consistent with several studies of genetic diversity in rice using SSR markers (Garris et al. 2005; Gealy et al. 2009; Londo and Schaal 2007). All loci were polymorphic compared to the total number of individuals, as indicated by the number of polymorphic alleles per locus (Table 2). The mean expected heterozygosity was 0.65 and the mean observed heterozygosity was 0.12 (Table 2). The mean PIC of the 24 SSRs was 0.61, ranging from 0.35 to 0.81 (Table 2). The high coefficient of inbreeding (f) confirmed the predominance of autogamy in the evaluated populations (Table 2).

The mean number of alleles in weedy red rice populations was 3.48 (Table 3). The proportion of polymorphic loci for the weedy red rice populations ranged from 0.92 to 1.00. The genetic diversity was 0.46 and the observed heterozygosity was 0.12 in all populations and at SSR loci (Table 3). Moreover, the value of f was high, with a mean of 0.77. However, the population Agudo 3 showed a low value for this parameter (Table 3), indicating a high rate of outcrossing with individuals from different populations or even with rice cultivars. This is corroborated by the high value of observed heterozygosity (0.3) in comparison with other populations (Table 3).

The results here indicate that weedy red rice populations from southern Brazil have a relatively high genetic diversity, although there is considerable variation among populations. This result differed from those for weedy rice populations in Arkansas (Shivrain et al. 2010b) and Japan (Kawasaki et al. 2009), where low genetic diversity was observed. Moreover, observations of genetic diversity of other weedy rice populations collected in the United States (Gealy et al. 2009), Italy (Jiang et al. 2012), and South Korea (Chung and Park 2010) were similar to those in the present study. A low genetic diversity is generally expected in natural inbreeding populations, due to the high degree of homozygosity. However, in weedy red rice populations from agricultural areas, the genetic diversity might be higher (Xia et al. 2011). Gene flow among

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Table 3. Sample size (*n*), mean number of alleles, alleles per polymorphic locus (P), expected heterozygosity or genetic diversity (He), observed heterozygosity (Ho), polymorphic information content (PIC), and inbreeding coefficient (*f*) of weedy red rice, wild rice, and rice cultivars.

Population	*n*	Mean no. of alleles	P	He	Ho	PIC	*f*
Arroio Grande 1	20	3.46	0.96	0.56	0.10	0.48	0.82
Arroio Grande 2	20	2.75	0.92	0.38	0.13	0.32	0.67
Arroio Grande 3	20	2.92	0.92	0.45	0.09	0.39	0.82
Santa Margarida do Sul 1	20	3.08	0.92	0.47	0.14	0.39	0.71
Santa Margarida do Sul 2	19	2.00	0.94	0.46	0.11	0.53	0.76
São Gabriel 1	20	3.50	1.00	0.48	0.08	0.42	0.83
São Gabriel 2	20	2.71	0.96	0.32	0.07	0.27	0.79
São Gabriel 3	17	2.46	1.00	0.53	0.07	0.58	0.86
São Gabriel 4	20	2.29	0.94	0.54	0.09	0.59	0.84
Cacequi 1	20	3.79	0.96	0.58	0.12	0.50	0.79
Cacequi 2	20	3.63	0.96	0.57	0.05	0.49	0.92
Cacequi 3	20	3.83	0.96	0.53	0.08	0.45	0.85
Cacequi 4	20	3.54	1.00	0.43	0.10	0.37	0.78
Cacequi 5	18	3.38	0.96	0.56	0.24	0.47	0.60
Cacequi 6	20	3.79	0.96	0.57	0.06	0.49	0.90
Santo Antônio da Patrulha 1	20	4.25	1.00	0.55	0.08	0.49	0.85
Santo Antônio da Patrulha 2	20	3.83	1.00	0.58	0.11	0.50	0.82
Campo Bom	20	3.67	1.00	0.45	0.10	0.38	0.78
Manoel Viana 1	20	3.79	1.00	0.54	0.22	0.47	0.59
Harmonia	20	3.54	0.96	0.43	0.09	0.38	0.79
Manoel Viana 2	20	4.08	1.00	0.60	0.14	0.52	0.78
Alegrete	20	3.25	0.88	0.45	0.10	0.39	0.79
Caverá	20	4.46	1.00	0.63	0.17	0.55	0.73
Agudo 1	20	4.46	1.00	0.67	0.17	0.55	0.77
Agudo 2	20	4.33	0.96	0.60	0.14	0.53	0.78
Agudo 3	20	3.54	0.92	0.55	0.30	0.46	0.48
Agudo 4	20	3.54	0.79	0.46	0.13	0.39	0.74
Population mean	—	**3.48**	**0.96**	**0.52**	**0.12**	**0.46**	**0.77**
Oryza glaberrima	1	1.27	0.27	0.27	0.27	0.18	0.00
Oryza longistaminata	1	1.39	0.39	0.39	0.39	0.36	0.00
Oryza rufipogon	1	1.39	0.39	0.39	0.39	0.18	0.00
Oryza glumaepatula	3	1.83	0.56	0.34	0.11	0.27	0.71
EL PASO L 144	3	1.00	0.00	0.00	0.00	0.00	0.00
IAS 12-9 FORMOSA	3	1.13	0.13	0.06	0.03	0.04	0.60
BR-IRGA410	4	1.22	0.17	0.08	0.04	0.09	0.50
IRGA 417	1	1.05	0.05	0.05	0.05	0.18	0.00
IRGA 422 CL	1	1.05	0.05	0.05	0.05	0.18	0.00
PUITÁ INTA CL	1	1.00	0.00	0.00	0.00	0.17	0.00
SATOR CL	1	1.60	0.60	0.60	0.60	0.52	0.00

agricultural fields is mainly caused by seed migration, resulting in a high genetic diversity in comparison with natural environments (Londo and Schaal 2007), and this factor is related to the results in this study.

The AMOVA of the weedy red rice plants showed that the genetic variation among and within populations was 26 and 74%, respectively (Table 4). The AMOVA indicated that the F_{ST} (Fixation index) value was 0.26, which is lower than those found in other weedy rice populations in other studies, which were 0.56 (Londo and Schaal 2007) and 0.44 (Gealy et al. 2009) in the United States, and 0.47 in Korea (Chung and Park 2010). Furthermore, a recent study performed in Italy for 24 weedy rice populations found an F_{ST} value of 0.25 (Jiang et al. 2012). The different values of genetic diversity within populations among these studies characterize differences in natural variability and the different levels of migration events mainly related to seed movement.

The weedy red rice populations evaluated in this study showed a high genetic variability. However, the high variation within populations reinforces the hypothesis of gene flow among populations. In weedy rice populations in Arkansas, the genetic diversity among populations was higher than within populations and a quarter of these populations shared alleles present in the rice cultivars (Shivrain et al. 2010a). In another study in South Korea, the genetic diversity between weedy red rice populations

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Table 4. Analysis of molecular variance of 27 weedy rice populations based on 24 single sequence repeat markers.

Source variation	df	Sum of squares	Variance components	Percentage of variation	*P*-value
Among populations	26	5,016.686	8.542	26%	0.001
Within populations	507	12,171.576	24.007	74%	0.001
Total	533	17,188.262	32.549		
Gene flow (Nm)[a]	0,7				

[a] $Nm = [(1/F_{ST}) - 1]/4$.

was similar to that observed in this study (Chung and Park 2010). The authors attributed the origin of certain populations of weedy red rice from South Korea to a cultivar whose use was discontinued (Chung and Park 2010). In the case of weedy rice populations from southern Brazil, two scenarios are possible regarding the causes of gene flow. The first scenario posits that it is related to seed migration, which is related to the widespread use of uncertified seeds that are often contaminated with weedy red rice by farmers in southern Brazil (Marchezan et al. 2001). Secondly, approximately 50% and 30% of the rice area at the time of sampling was cultivated with IRGA 417 and IRGA 422 CL rice cultivars, respectively. These cultivars are closely related, and IRGA 422 CL is resistant to imidazolinone herbicides. These characteristics create a bias, since these cultivars would share their alleles with populations geographically distant from each other, making the F_{ST} smaller than that observed in other studies. The estimate of gene flow (Nm) according to the infinite island model of Wright (1949), supports this hypothesis. Based on F_{ST}, the Nm was 0.7 (Table 4), which is higher than the values obtained in weedy red rice populations elsewhere in the world, which were estimated at 0.47 (Cao et al. 2006), 0.32 (Gealy et al. 2009), and 0.55 (Chung and Park 2010). The Nm here indicates that individuals have a mean of 0.7 migrants per generation. Theoretically, one individual migrant per generation, that is, $Nm = 1$, reduces the F_{ST} of a population by 80% (Templeton 2006). This means that only one individual migrant per generation can drastically narrow the genetic relationship between populations, indicating the large effect of the presence of weedy red rice seeds as contaminants of the crop seed on the genetic diversity within and among populations.

The result of the Mantel test showed no correlation between genetic and geographical distances of the evaluated weedy red rice populations. The coefficient of determination (R^2) was 0.06. Certain weedy rice populations that were geographically close (Table 1) were allocated to distinct subgroups in the dendrogram (Figure 1), such as the populations from São Gabriel, Santo Antônio da Patrulha, Cacequi, and Agudo. However, some populations that were geographically distant, such as Arroio Grande 2 and Cacequi 4, were allocated to the same subgroup (Figure 1). A low relationship between genetic and geographical distances can be related to the migration of weedy rice seeds among rice fields. In this case, paddy fields that are geographically distant can have weedy red rice populations with great genetic similarity due to the use of crop seeds contaminated with weedy red rice that originated from the same source. The result from the Mantel test indicated the absence of correlation between genetic and geographical distances, which is different from what has been observed in other studies in China (Xia et al. 2011) and the United States (Gealy et al. 2009). The lack of a direct relationship between genetic and geographical distances in the present study supports the hypothesis that significant seed migration between locations has occurred.

The PCA indicates a remarkably wide range of individuals from the same population across the three groups. For example, the population Cacequi 3 contained individuals in all three groups (Figure 2). However, a few populations, such as São Gabriel 4, showed all individuals clustered together in the same group (Figure 2). Moreover, populations from distant sites were allocated to the same groups (Table 1; Figure 2). This result corroborates the Mantel test, which indicated the absence of a significant correlation between genetic and geographical distances.

Genetic Relationship of Weedy Red Rice, Wild Rice, and Rice Cultivars. The dendogram shows three groups, where all weedy rice populations grouped together, except for the rice cultivars and wild *Oryza* species (Figure 1). Among wild species, the separation of *O. rufipogon* and *O. glumaepatula* from *O. longistaminata* and *O. glaberrima* was evident (Figure 1). Since *O. rufipogon* and *O. glumaepatula* are likely to have a similar origin

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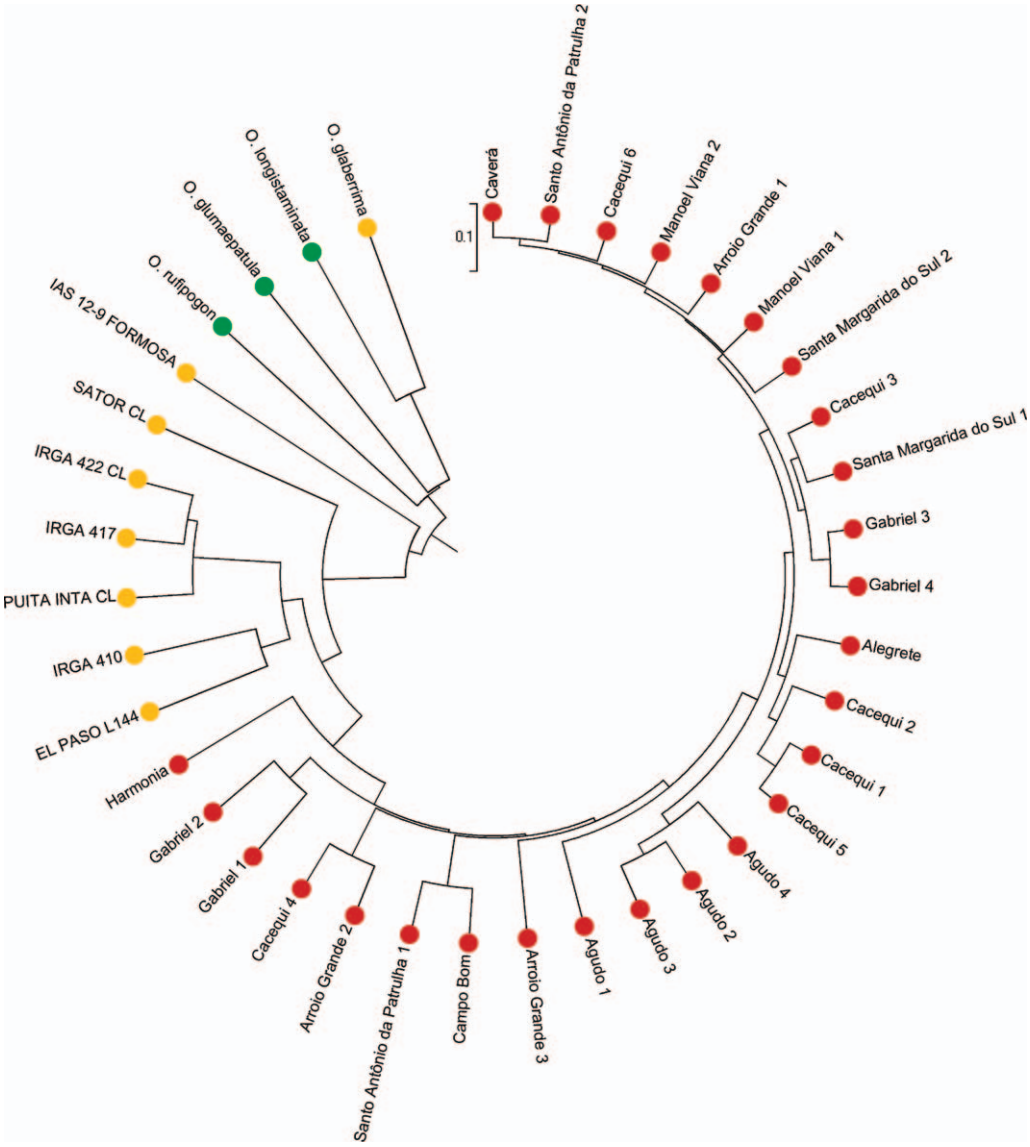

Figure 1. Clustering assessment obtained by the unweighted pair group method based on the genetic distance of 27 weedy rice populations (red dots), three wild rice species (green dots), and seven rice cultivars and *Oryza glaberrima* (yellow dots). (Color for this figure available in the online version of this paper.)

(Karasawa et al. 2007), they clustered together. The analyzed wild Oryza species—*O. rufipogon*, *O. glumaepatula, O. longistaminata*—and the cultivated *O. glaberrima* have the AA genome, similar to cultivated rice (Lu and Snow, 2005). Several studies have shown the occurrence of gene flow between O. *rufipogon* and cultivated rice (Olosfdotter et al. 2000; Wang et al., 2006). The high frequency of hybridization between cultivated rice and O. *rufipogon* indicates that this wild species is most likely to hybridize with cultivated rice, compared with other wild species. However, it is important to emphasize that O. *rufipogon* is not present in southern Brazil, but *O. glumaepatula* is native to the Amazon forest and western Brazil (Brondani et al. 2002). This region is located approximately 3,000 km from where most irrigated rice is cultivated in Brazil and where the samples of the present study were collected. The dendrogram also suggests that *O. glumaepatula* and the weedy red rice populations were not closely related (Figure 1). In this study, all the wild *Oryza* species—*O. rufipogon*, *O. glumaepatula, O. longistaminata*, and *O. glaberrima*—clustered separately from the weedy red rice populations, indicating the low probability of introgression among these wild species and weedy red rice.

Among rice cultivars, both SATOR CL and IAS 12-9 FORMOSA were isolated from the other cultivars (Figure 1). The *indica*-type cultivars IRGA 417, IRGA 422 CL, PUITA INTA CL, BR-IRGA 410, and EL PASO L144 grouped together, close to

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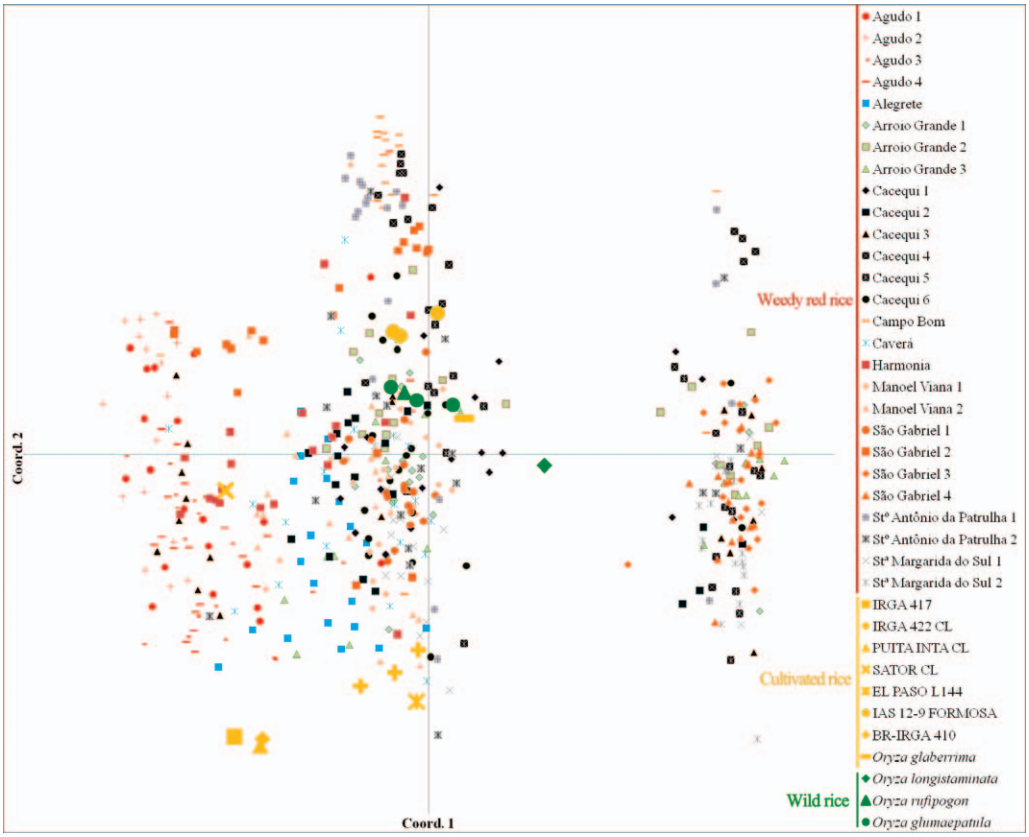

Figure 2. Principal component analysis of 27 weedy rice populations, three wild rice species, seven cultivars, and *Oryza glaberrima* based on the Nei's genetic distance (1978). (Color for this figure available in the online version of this paper.)

the group formed by weedy red rice populations (Figure 1). The proximity of these rice cultivars and the weedy red rice populations relates to the frequency and time of growth of these cultivars in the studied area. The cultivars BR-IRGA 410 and EL PASO L144 were developed from germplasm lines at International Center for Tropical Agriculture (CIAT)in Colombia that were subsequently selected in Brazil and Uruguay, respectively. These cultivars were widely used until the mid-1990s, when they were replaced by IRGA 417. The cultivar IAS 12-9 FORMOSA was originated from subspecies *japonica* parents and probably therefore grouped separately from the others. Both IRGA 422 CL and PUITA INTA CL have IRGA 417 as a progenitor (Goulart et al. 2012a; Livore et al. 2005) and were closely located within the same group (Figures 1 and 2). Moreover, the SATOR CL cultivar is a hybrid that has other genotypes as parents, but the origin of this cultivar is not known. In the study area, the use of the hybrid cultivar SATOR CL was very low; thus, gene flow from this cultivar is lower and its alleles are not shared with weedy rice populations in comparison with other cultivars. This might explain the higher genetic distance between this cultivar and weedy rice populations (Figure 1). Moreover, the clustering of weedy red rice populations close to other cultivars can be explained by the long-term use of BR-IRGA 410 and EL PASO L144 prior to 1990. Furthermore, it is evident that the importance of cultivar IRGA 417 as an allele donor for weedy red rice either derives directly by its widespread use since 1998 or derives indirectly as the main origin of the cultivars IRGA 422 CL and PUITA INTA CL (IRGA 2010; Livore et al. 2005).

The rice cultivars were distinctly situated in four different locations in the PCA (Figure 2). IRGA 417, IRGA 422 CL, and PUITA INTA CL clustered closely together but separately from weedy red rice groups, whereas SATOR CL was located apart from these cultivars. These results are in agreement with the dendrogram (Figure 1). Moreover, the old cultivars BR-IRGA 410 and EL PASO L 144 clustered close together, and IAS 12-9 FORMOSA was located separately from them. These results correspond with the genetic origin of these cultivars discussed previously. These cultivars were positioned in the intermediate cluster, together with individuals from various populations of weedy

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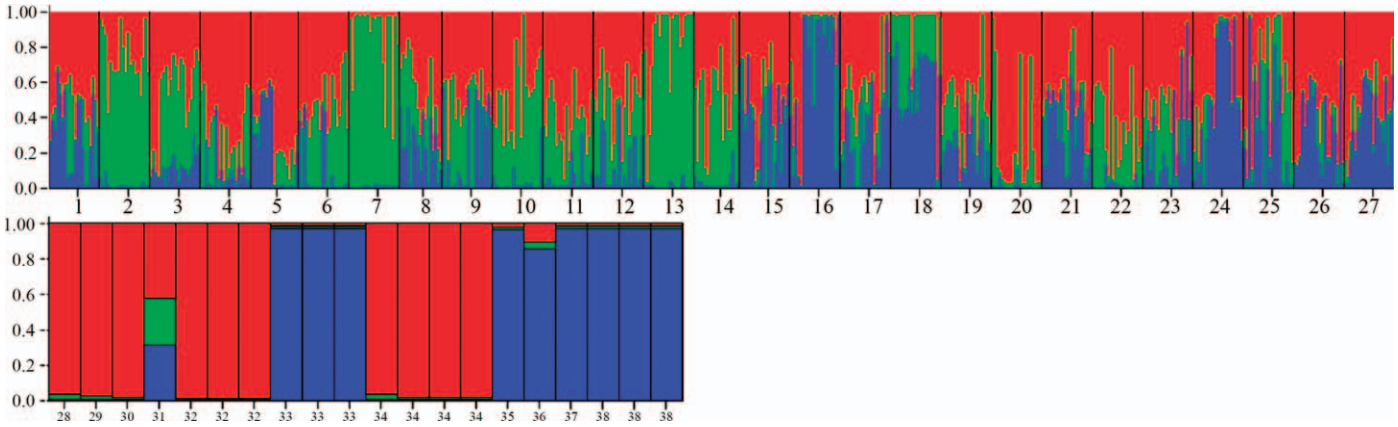

Figure 3. Bar plots derived from structure analysis of individuals from 27 weedy red rice populations (1 to 27), seven rice cultivars (18 to 34), three wild *Oryza* species (35 to 37), and *Oryza glaberrima* (38) as listed in Table 5. Each individual is represented by a single vertical bar assigned to the three potential genetic backgrounds (K groups, where red = K1, green = K2, and blue = K3). Longer bars indicate a greater contribution from a particular genetic background. The vertical axis represents the probability of the assignment. (Color for this figure available in the online version of this paper.)

red rice (Figure 2), indicating the occurrence of allele introgression from these cultivars to weedy red rice.

Population Structure and Herbicide Resistance Distribution. The results of population structure analysis indicated that the data set was optimally structured with K = 3, i.e., the population sampled might represent three possible genetic backgrounds (Figure 3). The proportion in which each population was assigned to each of the K clusters and the level of resistance to imidazolinone herbicide and characterization of *ALS* mutations are described in Table 5. The *indica*-type cultivars IRGA 417, IRGA 422 CL, PUITA INTA CL, BR-IRGA 410, and EL PASO L 144 were located in cluster K1 and the *japonica*-type cultivar IAS 12-9 FORMOSA was assigned to cluster K3 (Figure 3; Table 5). In southern Brazil, the *japonica*-type cultivars had not been grown since the introduction of dwarf *indica* cultivars. Some alleles of this type of cultivar apparently are still present in some weed red rice populations (Figure 3). The admixtured ancestry recognized in SATOR CL (Figure 3) is consistent with the hybrid characteristic of this cultivar. These findings are related to the known ancestry of the evaluated cultivars and this must be taken into consideration in the population structure analysis based on the selected K number (Chung and Park, (2010).

The results indicate the narrow genetic base of the cultivars used in southern Brazil in recent years. The continued use of certain cultivars can result in the selection of weedy red rice individuals with characteristics similar to the adopted cultivars (Cao et al. 2006). In addition, the recent use of herbicide-resistant cultivars and the consequent introgression of this trait into weedy red rice have contributed to the selection of weedy red rice plants more similar to the rice cultivars. This may explain the high level of similarity between weedy red rice individuals and the most recently grown rice cultivars that are imidazolinone-resistant in comparison with the older ones. The imidazolinone-susceptible populations São Gabriel 2, Cacequi 4, and Santo Antônio da Patrulha 1 displayed a K1 value lower than 0.20, indicating a low relationship with the imidazolinone-resistant rice cultivars (Table 5). Otherwise, the population Harmonia, which is also susceptible do imidazolinone herbicides, had a high relationship with the rice cultivars. In this population gene flow could be occurred from the non–herbicide-resistant cultivars, such as IRGA 417, which was largely grown before the recent adoption of the imidazolinone-resistant rice cultivars. The population Agudo 2 had a medium level of resistance and absence of the three *ALS* gene mutation (Table 5) indicating a possible independent evolution of resistance to the imidazolinone herbicides. However, the population of Campo Bom, with a moderately resistant phenotype, gave a value of 0.08 for cluster K1 (Table 5). This result might be explained by the presence of two individuals with a high level of resistance; one containing the mutation $Gly_{654}Glu$ and another containing $Ser_{653}Asn$ (data not shown).

The São Gabriel 1 and São Gabriel 2 populations, which are geographically close, showed a narrow genetic relationship and were closely grouped in the UPGMA analysis (Figure 1). However, they were

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Table 5. The proportion of assessment of 27 weedy red rice populations, seven rice cultivars, three wild *Oryza* species, and *Oryza glaberrima* defined by the structure analysis and the phenotypic and genotypic characterization of acetolactate synthase gene (*ALS* gene) mutations associated with resistance to imidazolinone herbicides.

Population		Cluster			Injury	Resistance Level[a]	*ALS* gene mutation
		K1 (red)	K2 (green)	K3 (blue)			
					%		
1	Arroio Grande 1	0.52	0.18	0.30	10.0	High	$Gly_{654}Glu$
2	Arroio Grande 2	0.26	0.72	0.02	10.4	High	$Gly_{654}Glu/Ser_{653}Asn$
3	Arroio Grande 3	0.47	0.41	0.12	3.0	High	$Gly_{654}Glu$
4	Santa Margarida Sul 1	0.68	0.24	0.08	7.3	High	$Gly_{654}Glu/Ala_{122}Thr$
5	Santa Margarida Sul 2	0.67	0.09	0.24	7.0	High	$Gly_{654}Glu$
6	São Gabriel 1	0.54	0.36	0.10	15.0	Medium	$Gly_{654}Glu$
7	São Gabriel 2	0.13	0.85	0.02	83.3	Low	—
8	São Gabriel 3	0.47	0.29	0.24	11.8	High	$Gly_{654}Glu$
9	São Gabriel 4	0.48	0.31	0.20	1.5	High	Outra/ $Gly_{654}Glu$
10	Cacequi 1	0.47	0.49	0.04	0.3	High	$Gly_{654}Glu$
11	Cacequi 2	0.62	0.33	0.05	4.2	High	$Gly_{654}Glu$
12	Cacequi 3	0.50	0.37	0.13	5.0	High	$Gly_{654}Glu/Ala_{122}Thr$
13	Cacequi 4	0.17	0.81	0.02	87.0	Low	—
14	Cacequi 5	0.49	0.47	0.04	2.3	High	$Gly_{654}Glu/Ala_{122}Thr$
15	Cacequi 6	0.49	0.14	0.37	14.3	High	$Gly_{654}Glu$
16	Sto Antônio Patrulha 1	0.20	0.12	0.69	88.8	Low	—
17	Santo Antônio Patrulha 2	0.47	0.21	0.32	0.0	High	$Gly_{654}Glu$
18	Campo Bom	0.08	0.32	0.60	64.3	Medium	$Gly_{654}Glu/Ser_{653}Asn$
19	Manoel Viana 1	0.55	0.23	0.22	14.5	High	$Gly_{654}Glu/Ser_{653}Asn$
20	Harmonia	0.77	0.21	0.02	98.5	Low	—
21	Manoel Viana 2	0.50	0.16	0.35	10.5	High	$Gly_{654}Glu$
22	Alegrete	0.65	0.32	0.03	5.0	High	$Gly_{654}Glu$
23	Caverá	0.52	0.23	0.25	9.5	High	$Gly_{654}Glu$
24	Agudo 1	0.40	0.09	0.52	5.5	High	$Gly_{654}Glu/Ser_{653}Asn$
25	Agudo 2	0.38	0.29	0.33	67.4	Medium	—
26	Agudo 3	0.61	0.08	0.32	16.3	High	$Gly_{654}Glu$
27	Agudo 4	0.54	0.09	0.37	10.8	High	$Gly_{654}Glu$
28	IRGA 417	0.96	0.03	0.01			—
29	IRGA 422 CL	0.97	0.02	0.01			$Gly_{654}Glu$
30	PUITÁ INTA CL	0.98	0.01	0.01			$Ala_{122}Thr$
31	SATOR CL	0.42	0.27	0.32			$Ser_{653}Asn$
32	EL PASO L144	0.98	0.01	0.01			—
33	IAS 12-9 FORMOSA	0.01	0.02	0.97			—
34	BR-IRGA 410	0.97	0.02	0.01			—
35	*Oryza glaberrima*	0.02	0.02	0.97			—
36	*Oryza longistaminata*	0.11	0.03	0.86			—
37	*Oryza rufipogon*	0.01	0.01	0.97			—
38	*Oryza glumaepatula*	0.01	0.01	0.97			—

[a] Low, > 85%; medium, 15–85%, high, > 15% of control.

assigned to different clusters in the structure analysis (Figure 3; Table 5). This might be explained by the fact that São Gabriel 1 is imidazolinone-resistant and São Gabriel 2 is susceptible (Table 5). Gene flow probably occurred from the resistant cultivars or resistant weedy red rice individuals to São Gabriel 1 individuals. The Cacequi 3, Cacequi 6, São Gabriel 4, and Santo Antonio da Patrulha 2 populations showed a high degree of admixture, meaning that their origin could not be attributed exclusively to one of the three inferred clusters (Figure 3). These results indicate that migration events among the evaluated populations occurred, probably via weedy red rice seeds. This is related to the use of rice seeds contaminated with weedy red rice. A study performed in 1998 with 117 southern Brazilian rice farmers concluded that less than 10% of farmers used certified seeds (Marchezan et al. 2001). In this evaluation, 83% of rice seeds samples were contaminated with weedy rice, ranging from 1 to 20 seeds per sample (Marchezan et al. 2001). In another study conducted in 2008 in this region, 38% of the rice seed samples contained seeds of imidazolinone-resistant weedy red rice (Ferreira et al. 2009).

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Together with the results of the present study, this suggests that the use of contaminated rice seeds is the main explanation for the origin and distribution of the imidazolinone-resistant weedy red rice in southern Brazil. In addition, other factors such as bird dispersion and machinery movement among contaminated fields might contribute to weedy red rice seed migration between paddy fields. However, several imidazolinone-susceptible weedy red rice populations with little genetic similarity to the resistant populations were found, suggesting that gene flow control practices such as planting seeds free of weedy rice contaminants may have been adopted for these paddy fields.

Implications for Weedy Red Rice Management. The results obtained by AMOVA, dendrogram, and population structure analysis indicated the occurrence of gene flow between resistant rice cultivars and weedy red rice as well as intensive weedy red rice seed migration among populations. Thus, this might be the predominant source of imidazolinone resistance of the weedy rice in southern Brazil. The occurrence of several populations containing more than one mutation conferring resistance to *ALS* inhibitors (Table 5) is notable. This was also found in another study, in which weedy rice populations possessed three distinct mutations in the *ALS* gene (Roso et al. 2010), indicating the rapid stacking of genes related to herbicide resistance. The information generated by these studies is important because it provides insights into how weedy rice populations have evolved in modern rice field systems. These results highlight the problem of gene flow from resistant rice cultivars, resulting in a limitation of this technology due to migration of the resistance gene to weedy red rice. However, several transgenic technologies are being developed for glufosinate ammonium resistance (Gealy et al. 2003; Michiels and Johnson 2001), glyphosate resistance (Hu et al. 2009; Zhou et al. 2006), and protoporphyrinogen oxidase inhibitors (Jung et al. 2010). In addition, new natural-mutant rice cultivars resistant to herbicides other than the ALS inhibitors are currently being developed (Merotto et al. 2013). Nevertheless, the use of these resistant cultivars might result in gene flow of the herbicide-resistant trait to weedy red rice in a similar way to that of imidazolinone-resistant cultivars. Some biotechnology-based strategies have been proposed to reduce gene flow in rice. The main examples are cleistogamy, maternal inheritance, and the incorporation of the resistance gene together with an antidwarfism, antishattering trait and susceptibility to herbicide genes (Al-Ahmad and Gressel 2006; Gressel and Valverde 2009; Lin et al. 2008; Yoshida et al. 2007). In addition, farmers' attitudes must be improved regarding conventional methods of weedy rice control, such as crop rotation, rotation of rice establishment systems, burn-down herbicide application, elimination of weedy red rice escapees, and, most importantly, the use of rice seeds free of weedy rice contaminants.

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